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A Novel Hepatitis B Virus Variant S 129 (Gln→Leu): Lack of Correlation Between Antigenicity and Immunogenicity

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A point mutation has been detected in the "a" determinant of hepatitis B surface antigen (HBsAg) in an infant immunised with hepatitis B vaccine after exposure to hepatitis B virus (HBV). This A-to-T point mutation at nucleotide 540 resulted in a glutamine-to-leucine substitution at amino acid residue 129 (129L). The S gene fragment (nucleotide 58–1072) of this isolate was cloned and used to substitute the wild-type S gene in a plasmid (p3.8II), containing 1.2 copy of full-length HBV genome with expression and replication efficiency. This plasmid p3.8II-129L was used to transfect HepG2 cells. HBsAg expressed by p3.8II-129L showed higher binding efficiency compared with the original plasmid containing the wild-type clone. A panel of 24 anti-HBs monoclonal antibodies (MAbs) was used to characterise the binding efficiency of HBsAg expressed by p3.8II-129L. Eighteen showed higher binding to the antigen, whereas HBsAg expressed by p3.8II-145R gave a consistently lower absorbance or were negative. Surprisingly, when the immunogenicity of plasmid constructs was used for DNA immunisation in Balb/c mice, the anti-HBs response induced by p3.8II-129L was significantly lower than that of the wild-type p3.8II. The lack of correlation between the antigenicity profile (binding of expressed HBsAg to anti-HBs in vitro), and the immunogenicity (induction of anti-HBs by plasmid DNA in vivo) of HBsAg with leucine substitution at position 129 indicates that biological characteristics other than the binding efficiency of HBsAg to anti-HBs could occur in HBsAg variants. These different aspects of the biological characteristics of S mutants merit further investigation. ***J. Med. Virol. 59:424–430, 1999.***

KEY WORDS: hepatitis B virus; mutants; DNA immunisation; HBsAg "a" determinant

INTRODUCTION

The genome of hepatitis B virus (HBV) is approximately 3.2 kb, with a unique double-stranded circular DNA molecule that is partially single stranded. There are four open reading frames in the long DNA strand. These encode for the viral envelope (S/Pre-S gene), viral nucleocapsid (C/Pre-C gene), viral polymerase (P gene), and a multifunctional transactivating X protein (X gene), respectively. Because the genome of HBV is very compact, these open reading frames overlap, of significance when attesting the importance of individual point mutations in separate genes. We and others [Carman et al., 1990; Fujii et al., 1992; Harrison et al., 1994; Howard et al., 1994; Wen et al., 1995; He et al., 1998] have described S gene mutants in infants receiving either hepatitis B vaccination or vaccine together with HBIg. Molecular epidemiological studies to date indicate that failure of protection in such infants does not seem to be the result of vaccine escape mutants [Ngui et al., 1997; He et al., 1998]. Extensive characterisation of these hepatitis B surface antigen (HBsAg) "a" determinant mutants is often lacking, with the result that it is unclear whether or not these mutants possess other important characteristics that may determine the outcome of HBV infection. We report the lack of correlation between antigenicity and immunogenicity of an HBV variant in an infant associated with an amino acid change from glutamine to leucine at residue 129 in the HBsAg "a" determinant. Our data confirm the importance of attesting fully the properties of variant HBsAg, both in vitro and in vivo. The role of such variants in the infective process has considerable diagnostic and clinical implications.

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MATERIALS AND METHODS

Sera and Amplification of the HBV S Gene

Sera from infants born to HBV DNA-positive mothers who became HBsAg positive despite postexposure immunisation with hepatitis B vaccination (30 μg plasma-derived HBsAg per dose, three doses given at birth, and 1 and 6 months after birth, respectively) were collected, following approved ethical guidelines. Serum DNA was extracted as described previously [He et al., 1998], and the S gene amplified using the following primers: Forward, Seq 1 (5′-CCTGCTGGTGGCTCC AGTTC-3′ nucleotide 58–77); Reverse, Seq 12 (5′-CATGCATACAAAGGCATC AAGGC-3′ nucleotide 1072–1050). Ten microliters of DNA template, 1 μM of each of the primers, 0.2 mM of 4× dNTP, and 2.5 U of Taq DNA polymerase (Boehringer-Mannheim, Mannheim, Germany) were mixed in a final volume of 100 μl. The polymerase chain reaction (PCR) was carried out at 94°C for 50 sec, 55°C for 1 min, and 72°C for 1 min, for 32 cycles, with an extension of 10 min at 72°C after the last cycle.

PCR Product Purification, Cloning, and Sequencing

The PCR product was first checked for positivity by agarose gel electrophoresis, and then purified using Prep-A-Gene (Bio-Rad, Cambridge, MA). The PCR product was cloned into the pGEM vector using T-A cloning kit (Promega, Madison, WI). DNA sequencing was performed by the dideoxy-chain termination procedure, using the *fmol* sequencing kit (Promega). Two isolates having mutations were isolated from two infants infected persistently with HBV. The first with a mutation found at nucleotide 540 (A→T), predicted as having an amino acid substitution from glutamine to leucine at residue 129; and the second displayed a mutation at nucleotide 587 (G→A), predicted as containing a substitution from glycine to arginine at residue 145. Plasmid clones containing the mutant S genes were referred to as pGEM-129L and pGEM-145R, respectively.

Construction of Recombinant Plasmids p3.8II-129L and p3.8II-145R

Constructs were prepared starting with a recombinant plasmid p3.8II (a gift from Professor Yuan Wang, Shanghai Institute of Biochemistry, Academia Sinica) contained a more than full-length (1.2) copy of a wild-type *adr* HBV genome inserted in vector plasmid pBS+. Figure 1 outlines the cloning strategies for constructing p3.8II-129L and p3.8II-145R. The composition of both was confirmed by automated DNA sequencing.

Transfection of HepG2 Cells

Plasmid DNA from p3.8II, p3.8II-129L, and p3.8II-145R were purified using anion exchange columns (Midi Prep, Qiagen, Germany). Each preparation was used to transfect HepG2 cells as reported previously [Wen et al., 1989]. In brief, transfection was carried out by the calcium phosphate precipitation method, with 20 μg of constructed plasmid DNA and 10 μg of reporter plasmid expressing secreted alkaline phosphatase (SEAP) per 60-mm-diameter plate. Vector pBS+ without HBV insert was used as a mock control in transfection. After transfection, supernatant from transfected cells was collected every 3–4 days up to day 18. Supernatants were stored frozen at −70°C until assayed for HBsAg by EIA (Abbott, North Chicago, IL). Hepatitis B e antigen (HBeAg) expression was measured only on day 3. Each transfection experiment was performed twice and two plate of cells were transfected with the same sample each time.

Assay for HBsAg by 24 "a" Determinant MAbs

Twenty-four different "a" MAbs (recognising epitopes not identified) were provided by the Hepatitis Laboratory, National Institute for the Control of Pharmaceutical and Biological Products, China and were used in an enzyme-linked immunosorbent assay (ELISA) test against supernatant collected 3 days after transfection. Absorbance were determined by an ELISA Reader (Bio-Rad).

DNA Immunisation of Mice

DNA inocula were prepared from each plasmid preparation by anion exchange column (Maxi Prep, Qiagen, Germany). DNA was resuspended in endotoxin-free sterile physiological saline and used to immunise female Balb/c mice (weight 16–18 g, pathogen free, provided by the Shanghai Experimental Animal Center, Chinese Academy of Sciences, China). All DNA samples were checked for endotoxin prior to injection (less than 0.25 endotoxin U/μg). Groups of five animals were injected according to the protocols of Davis et al. [1993]. Briefly, mice were first anaesthetised with sodium pentobarbital and then 100 μg of plasmid DNA were injected into the tibialis anterior muscles of the hind legs (each leg 50 μg). Mice were boosted with same dose of plasmid DNA 6 weeks after the first injection, and sera were collected at 4, 6, 9, and 11 weeks after the first inoculation, respectively. Sera were examined for anti-HBs using a modified anti-HBs detection kit (KEHUA Co., Shanghai, China) [Qu et al., 1998].

RESULTS

Sequence Analyses of the S Genes Cloned From the 129L and 145R Variants

The S gene sequences and the predicted amino acid sequences of the two 129L, 145R variants were compared with the wild-type HBV (Fig. 2A and 2B). The mutation at 129L has not been reported previously (deposited at EMBL database, accession number AJ 005283). In addition to the point mutations detected at residue 129, there are four additional amino acid differences predicted between the mutant 129L and the

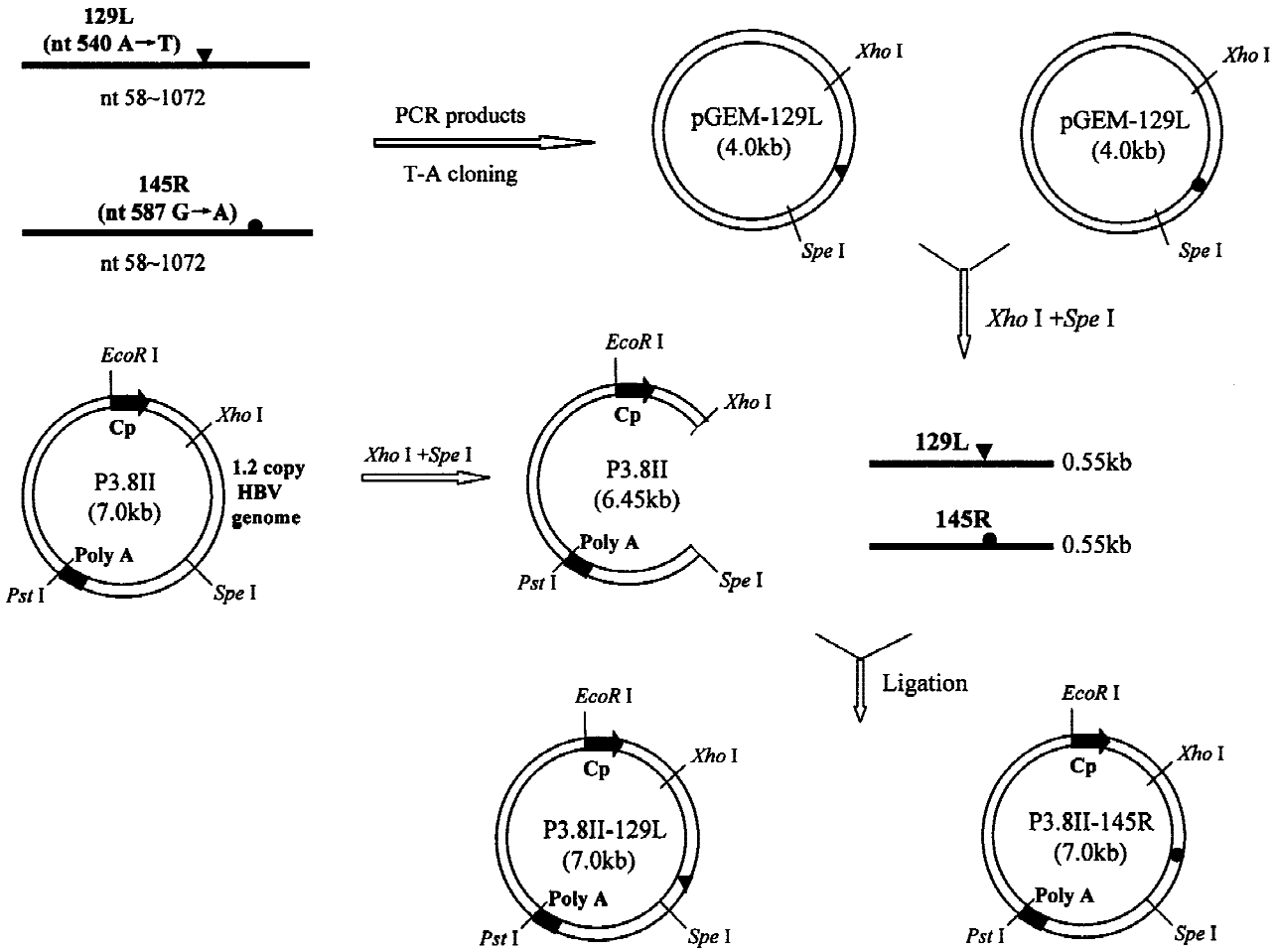

Fig. 1. Construction of hepatitis B virus (HBV) recombinant plasmids p3.8II-129L and p3.8II-145R. Amplified polymerase chain reaction (PCR) products (HBV DNA nucleotide [nt] 58–1072) from serum samples with S gene mutations at 540 (A→T) or 587 (T→A) were first cloned separately into pGEM vector by T-A cloning, followed by digestion with *Xho*I and *Spe*I restriction enzymes, to yield two fragments (0.55 kb, HBV DNA nucleotide 129–679 and 6.45 kb, vector pBS+ and part of HBV genome excluding nucleotide 129–679). The 0.55-kb fragment was used to substitute the corresponding wild-type HBV S gene fragment in p3.8II, and the resulting clones were named p3.8II-129L and p3.8II-145R respectively. Cp indicates core promoter region of HBV; p3.8II is a vector plasmid pBS+ with a 1.2 copy of wild-type HBV genome insertion.

wild-type virus (at amino acids 3, 112, 126, and 160). For 145R mutant, there are three predicted additional amino acid substitutions, which are at the 66, 118, and 150 residues.

HBsAg and HBeAg Expression in Transfected HepG2 Cells

The level of HBsAg expression by each transfectant was adjusted based on SEAP expression assay, and is shown in Figure 3. Significant higher HBsAg absorbance was found in cells transfected with plasmid p3.8II-129L. Because the pre-Core/Core gene is identical in plasmids p3.8II, p3.8II-129L, and p3.8II-145R, HBeAg expression of these three plasmid constructs was used as an internal reference for monitoring the overall concentration of protein expression. As indicated in Figure 4, the amount of HBeAg in the supernatant of cells transfected with all three plasmids was similar (*t*-test, $P > .05$); therefore, the higher binding efficiency of HBsAg expressed by 129L is of significance (run test, $P < .05$).

Binding Efficiency of HBsAg Expressed by p3.8II-129L

The binding to 24 "a" MAbs of HBsAg expressed by p3.8II-129L compared with the wild-type virus is shown in Figure 5. A higher binding efficiency was found against 18/24 MAbs, whereas MAbs #4, #13, and #20 showed a decrease in binding, suggesting that these three MAbs are probably directed to epitope at or around amino acid 129.

Immunogenicity of p3.8II-129L in Balb/c Mice

The anti-HBs antibody response was measured in mice immunised with the three plasmid DNA constructs (Table I). In mice immunised with p3.8II-145R DNA, anti-HBs antibodies were detected at a very low level or not at all. Unexpectedly, however, p3.8II-129L

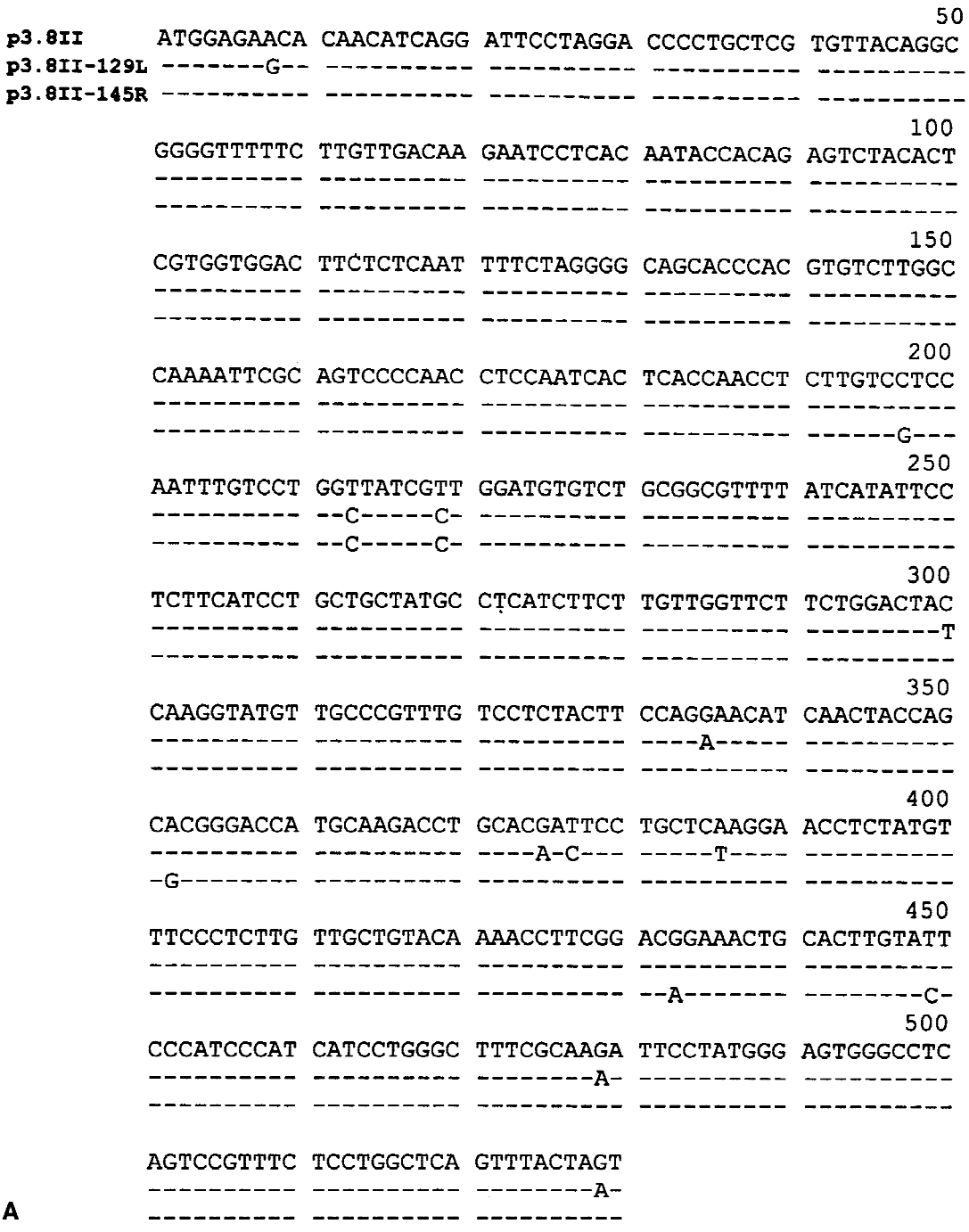

Fig. 2. **A:** Alignment of the nucleotide sequences of hepatitis B virus (HBV) S gene (1–530 nucleotides) of p3.8II, p3.8II-129L, and p3.8II-145R. Dashes represent nucleotides identical to those in the p3.8II wild type S gene.

DNA did not induce a higher titre of anti-HBs antibodies compared with mice injected with the wild-type virus DNA.

DISCUSSION

Carman and Mimms [1997] have proposed the existence of two types of HBV variations based on changes observed in the S gene. Class I refers to naturally occurring variants and class II encompasses those mutants selected for after vaccination and HBIg administration. Both of the isolates (129L and 145R mutants) reported here were from infants who underwent hepatitis B vaccination, and thus should be classified as class II variants, which most likely would show mutations within the neutralising epitope domain. The "a" determinant of HBsAg has been recognised as an important neutralising epitope. A number of mutations, e.g. mutations leading to amino acid substitution at

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                    10          20          30          40
p3.8II      MENTTSGFLG  PLLVLQAGFF  LLTRILTIPQ  SLHSWWTSLN
p3.8II-129L --S-------  ----------  ----------  ----------
p3.8II-145R ----------  ----------  ----------  ----------

                    50          60          70          80
            FLGAAPTCLG  QNSQSPTSNH  SPTSCPPICP  GYRWMCLRRF
            ----------  ----------  ----------  ----------
            ----------  ----------  -----R----  ----------

                    90         100         110         120
            IIFLFILLLC  LIFLLVLLDY  QGMLPVCPLL  PGTSTTSTGP
            ----------  ----------  ----------  -E--------
            ----------  ----------  ----------  -------A--

                   130         140         150         160
            CKTCTIPAQG  TSMFPSCCCT  KPSDGNCTDI  PIPSSWAFAR
            -----T--L-  ----------  ----------  ---------K
            ----------  ----------  ----R----T  ----------

                   170
            FLWEWASVRF  SWLSLL
            ----------  ------
B           ----------  ------
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Fig. 2. Continued. **B:** Alignment of the deduced amino acid sequences of HBV S protein (1–176 amino acid) of p3.8II, p3.8II-129L, and p3.8II-145R. Dashes represent amino acids identical to those in the p3.8II wild-type S protein.

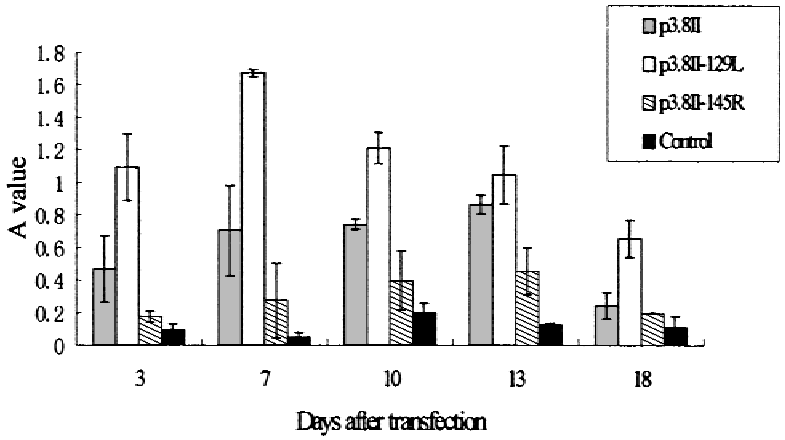

Fig. 3. Kinetic detection of hepatitis B surface antigen (HBsAg) in supernatants of transfected cells. Cells were transfected separately with plasmids p3.8II (▨), p3.8II-129L (□), p3.8II-145R (▧), and control vector pBS+ (■). Supernatants were collected on 3, 7, 10, 13, and 18 days, and assayed for HBsAg at 1:40 dilution using Abbott EIA commercial kits (Chicago, IL). Error bar represents SD (standard deviation). The higher binding efficiency of HBsAg expressed by 129L is of significance (Run test, $P < .05$).

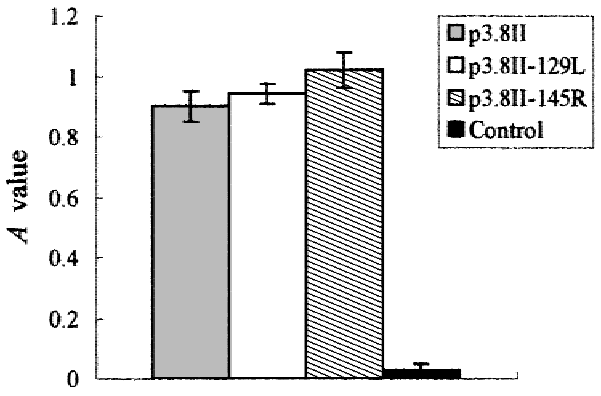

Fig. 4. Detection of hepatitis B e antigen (HBeAg) in supernatants of cells transfected with p3.8II (▨), p3.8II-129L (□), p3.8II-145R (▧), and vector pBS+ (■) 3 days after transfection. Assay of HBeAg was conducted in 1:4 dilution using Abbott EIA commercial kit (Chicago, IL). Error bar represents SD (standard deviation). The level of HBeAg in the supernatant of cells transfected with all three plasmids was similar (*t*-test, $P > .05$).

residues 145 Arg [Carman et al., 1990], or amino acid insertion at 122, 123 residues [Hou et al., 1995], result in a decrease or loss of HBsAg reactivity using the present available HBsAg diagnostic reagents. Uniquely, an increase in the binding efficiency of HBsAg has been reported in a variant at residue 126 (threonine→serine mutant) [Fang et al., 1998]. Recently, the change of lysine to glutamic acid at residue 141 has been associated with enhanced binding to MAbs [Karthigesu et al., 1999].

In this study, we have concentrated on studying the properties of an unreported glutamine-to-leucine substitution at residue 129. To study the antigenic reactivity of HBsAg expressing the 129L change, the variant S gene was cloned and the resulting plasmid construct used for in vitro expression of HBsAg and DNA immunisation mimicking natural virus infection. We did not use an exogenous promoter in these constructs but made use of the virion promoter per se, by substi-

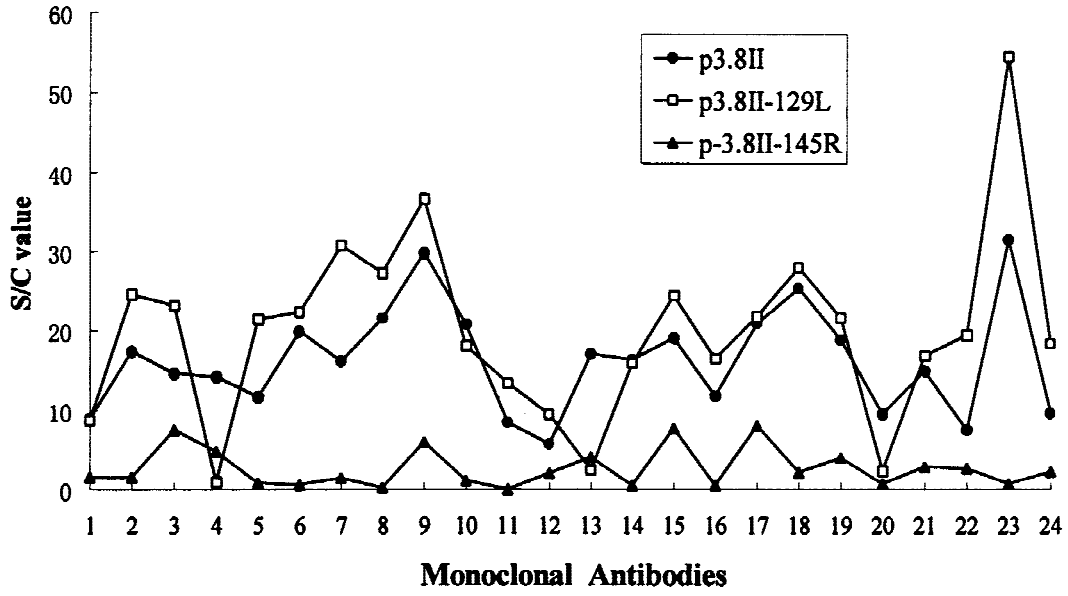

Fig. 5. Binding efficiency of hepatitis B surface antigen (HBsAg) from cells transfected with p3.8II (●), p3.8II-129L (□) and p3.8II-145R (▲), 6 days after transfection, was assayed against 24 monoclonal antibodies (MAbs). These MAbs (numbered in the abscissa) are directed against "a" determinant of HBsAg. After titration and optimisation, equal concentration of each MAb was used to coat 96-well plates and the supernatants of plasmid-transfected cells assayed for HBsAg in an enzyme-linked immunosorbent assay (ELISA) test. S/C value indicates the Absorbance of HBsAg divided by the Absorbance of control (supernatant of cells mock transfected with vector pBS+).

TABLE I. Kinetics of Anti-HBs IgG Response in Balb/c Mice Immunised With HBV DNA Recombinant Plasmids

	A value of anti-HBs in sera of mice[a]			
Weeks[b]	pBS+ Vector[c]	p3.8II[c]	p3.8II-129L[c]	p3.8II-145R[c]
4	0.066 ± 0.015	0.096 ± 0.029	0.071 ± 0.019	0.073 ± 0.021
6	0.199 ± 0.060	0.354 ± 0.185	0.134 ± 0.025	0.108 ± 0.005
9	0.159 ± 0.055	1.005 ± 0.455	0.113 ± 0.054	0.117 ± 0.057
11	0.195 ± 0.066	1.148 ± 0.797	0.267 ± 0.215	0.089 ± 0.019

HBV, hepatitis B virus.
[a]Mice sera were diluted to 1:100 and analysed for anti-HBs using a modified anti-HBs detection kit (KEHUA Co., Shanghai, China) [Qu et al., 1998]. Absorbance (*A*) was determined by ELISA Reader (Bio-Rad, USA) at 492 nm. Results are shown as mean ± SD.
[b]Weeks after the first immunisation.
[c]As indicated in Fig. 1.

tuting the wild-type HBsAg gene in p 3.8II with the 129L mutated S gene. In addition, the HepG2 cell line was used for transfections owing to their hepatocyte properties. To exclude any possible structural modification on the expressed HBsAg, a tag to the mutated gene was not added. Alternatively, because the pre-Core/Core gene of p3.8II, p3.8II-129L, and p3.8II-145R is identical, the level of HBeAg expressed after transfection could be used as an internal reference marker of protein expression, thus making the level of HBsAg expression comparable between cultures of HepG2 cells transfected with each of the plasmids. Based on these criteria, the results demonstrate clearly that the expressed HBsAg containing the 129L substitution binds antibodies with greater efficiency than wild-type virus. Similar results were obtained using a panel of MAbs, save that three antibodies reacted with much lower efficiency. This finding suggests that these antibodies could be directed to an epitope spanning residue 129. The much lower binding efficiency of 145R mutant is expected, as it has been demonstrated that this mutant leads to loss of anti-HBs antibody binding [Carman et al., 1990].

The plasmid DNA immunisation result is intriguing. Because the HBsAg expressed by 129L mutant showed higher binding efficiency with anti-HBs, one would expect higher immunogenicity of this mutant. To our surprise, this was not the case; a lower titre of anti-HBs response was observed. We preferred DNA immunisation rather than using purified HBsAg protein for immunisation, because DNA immunisation allows antigen to be presented via the endogenous pathway [Donnelly et al., 1997], a mechanism similar to natural infection. Thus the immune responses would resemble more closely those in natural infection. With regard to the low anti-HBs response detected in mice injected with the 145R mutant DNA, this finding is not surprising, as the assay kit was coated with wild-type HBsAg, and thus would not recognise anti-HBs antibodies induced by the 145R mutant. Compared with our previous study of wild-type HBV DNA vaccination, anti-HBs induced by vaccination with p3.8II was much lower in titre than that induced by vaccination with plasmid DNA using a cytomegalovirus early promoter (data not shown). This difference could be due to the position of immune-stimulating sequences or CpG [Krieg et al., 1995] in the p3.8II vector was not being optimum for the enhancement of immune responses. Because the mouse is used routinely for assessing the potency of hepatitis B vaccines, we presume that our data obtained from DNA immunisation in mice is relevant to predicting responses in human.

The question has been raised as to "whether all s-variants are clinically of equal importance, e.g., in terms of development of chronic sequelae of HBV infection" [Hess et al., 1997]. Our results indicate that in this regard, the 129L S mutant, although it expressed higher binding efficiency for anti-HBs antibodies, its immunogenicity in inducing anti-HBs antibodies was lower than that of the wild-type virus. It may be predicted that this variant strain would probably persist and result in chronic infection in an infected individual as a result of a low antiviral immune response.

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